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AS52/GPT Mammalian Mutagenesis Assay

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6. AUTHOR(S)
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5d. PROJECT NUMBER
ILS A073-003

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96-01

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Integrated Laboratory Systems
800-12 Capitol Drive
Durham, NC 27713

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U.S. Amy Center for Health Promotion and Preventive Medicine
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14. ABSTRACT
The trifuloromethane in either the presence or absence of metabolic activation did not induce a significant increase in mutant frequency at the gpt locus in cultured AS52 cell.

15. SUBJECT TERMS
AS52 cells, GPT Mammalian Mutagenesis Assay, FE-13, Trifluoromethane.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
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Integrated Laboratory Systems

STUDY TITLE
AS52/GPT MAMMALIAN MUTAGENESIS ASSAY

Contract No.
DAAD05-91-C-0018

ILS Project No.
A073-003

Test Substance
FE-13

ILS Repository No.
96-01

Final Report Date
May 10, 1996

Sponsor
U.S. Army CHPPM
Bldg E-2100
Aberdeen Proving Ground, MD 21005

Testing Facility
Integrated Laboratory Systems
800-12 Capitola Drive
Durham, NC 27713

P.O. Box 13501
Research Triangle Park, NC 27709

QUALITY ASSURANCE INSPECTION STATEMENT

ILS Project No.: A073-003
Test substance ID: FE-13
ILS Repository No.: 96-01
Study Title: AS52/GPT Mammalian Mutagenesis Assay

This study was inspected by one or more persons of the Quality Assurance Unit of Integrated Laboratory Systems, Research Triangle Park, NC, and written status reports were submitted on the following dates:

Inspection/Audit	Date Performed	Date Reported to Study Director/Management
Study Protocol	2/20/96	2/20/96; 2/20/96
Plate Counts	3/25/96	3/27/96; 4/01/96
Dosing	4/04/96	4/08/96; 4/12/96
Data Audit	5/8-9/96	5/09/96; 5/10/96
Final Report Audit	5/09/96	5/09/96; 5/10/96

Kaye Cummings
Kaye Cummings, B.S.
Quality Assurance Officer

5/10/96
Date

CERTIFICATION OF GOOD LABORATORY PRACTICES

ILS Project No.: A073-003
Test substance ID: FE-13
ILS Repository No.: 96-01
Study Title: AS52/GPT Mammalian Mutagenesis Assay

This study was conducted in accordance with Good Laboratory Practice regulations as promulgated by the U. S. Environmental Protection Agency (40 CFR Part 792) except for the following: the purity of the positive controls was not provided by the manufacturer.

Paul Andrews, M.S.
Study Director

5/10/96
Date

CERTIFICATION OF CONTRACT COMPLIANCE

ILS Project No.: A073-003
Test substance ID: FE-13
ILS Repository No.: 96-01
Study Title: AS52/GPT Mammalian Mutagenesis Assay

The contractor, Integrated Laboratory Systems, hereby certifies that, to the best of its knowledge and belief, the technical data delivered herewith under Contract No. DAAD05-91-C-0018 is complete, accurate, and complies with all requirements of the contract.

R. Tice for

Raymond R. Tice, Ph.D.
Vice President, Research & Development

5/10/96
Date

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FINAL REPORT

1.0 Study Title:

AS52/GPT Mammalian Mutagenesis Assay

2.0 Study Identification:

ILS Project No. A073-003
Contract No. DAAD05-91-C-0018

3.0 Purpose of the Study:

To evaluate the ability of FE-13 and/or its metabolites to induce gene mutations in the guanine phosphoribosyl-transferase (*gpt*) locus of cultured AS52 Chinese Hamster Ovary cells in the absence or presence of metabolic activation.

4.0 Names and Addresses of Sponsor and Testing Facility:

4.1	Sponsor:	U.S. Army CHPPM Bldg E-2100 Aberdeen Proving Ground, MD 21005
4.2	Testing Facility:	Integrated Laboratory Systems
	Shipping Address:	800-12 Capitola Drive Durham, NC 27713
	Mailing Address:	P.O. Box 13501 Research Triangle Park, NC 27709

5.0 Study Dates:

Study Initiation Date:	February 20, 1996
Experimental Start Date:	February 22, 1996
Experimental Termination Date:	May 8, 1996
Study Completion Date:	May 10, 1996

6.0 Primary Study Personnel:

Paul W. Andrews, M.S., Study Director
Leslie Hill, B.S., Research Assistant

7.0 Test substance:

7.1 Identification: FE-13

7.2 Physical description and composition: gas

7.3 Properties of the Test substance:

7.3.1 Compound Characterization: Determination of test substance stability and test substance characteristics is the responsibility of the sponsor. Information on the test substance including its method of synthesis, analysis, physicochemical characteristics, and bulk stability is retained on file by the sponsor.

7.3.2 Storage Conditions: The test substance was stored at room temperature in the chemical repository or treatment laboratory. Stability under these conditions has been demonstrated by the Sponsor and documentation is on file with them. Normal safety precautions appropriate for potential mutagens were followed when handling the test substance. A material safety data sheet was not provided.

8.0 Test System:

8.1 Test System Justification: AS52 cells contain a single, functional, stably integrated copy of the *Escherichia coli* xanthine-guanine phosphoribosyl transferase (XPRT) gene (*gpt*). Mutations at the *gpt* locus can be detected as 6-thioguanine resistant (6-TG^r) colonies under conditions identical to those for detecting mutations at the *hprt* locus in CHO-K1-BH4 cells (1). However, in contrast to the *hprt* assay, the AS52/*gpt* assay is able to detect agents which induce primarily small and large deletion mutations in addition to point mutations (1,2). This ability to detect clastogens in addition to point mutagens results in an assay equal in sensitivity to the mouse lymphoma TK$^{+/-}$ assay while retaining the technical simplicity of the CHO-K1-BH4 *hgprt* assay (3). The AS52/*gpt* assay has been used to study a wide range of mutagens and clastogens, including radiation, a wide variety of chemicals, and complex mixtures.

8.2 AS52 Cell Line: The AS52 cell line, supplied by Dr. K.R. Tindall of the US National Institute of Environmental Health Sciences, is a proline auxotroph with a modal chromosome number of 20, a population doubling time of approximately 14 hours and a cloning efficiency (CE) normally greater than 80%. Cells were cultured in Ham's F-12 medium with 5% fetal bovine serum plus additives (xanthine, adenine, thymidine, mycophenolic acid, and aminopterin) at 37 ± 1°C in a humidified atmosphere of 5 ± 1% CO_2 in air.

9.0 Experimental Design:

9.1 Preparation of the Dosage Formulation: As specified by the sponsor, test substance dosing mixtures were freshly prepared in air on the day of treatment.

9.2 Method of Administration and Justification: On the day of treatment, culture flasks were refed with a reduced volume of media (2.5 ml total) in order facilitate diffusion of the gaseous test substance to the cells. Flasks were sealed in plastic tedlar bags, the ambient air removed by vacuum, and the test substance doses introduced via a stainless steel valve stem.

9.3 S9 Activation System: Immediately prior to use, freshly thawed aliquots of Aroclor 1254-induced rat liver homogenates (S9 fraction) (Molecular Toxicology, Rockville, MD) were mixed with a sterile cofactor pool. The S9 reaction mixture was stored on ice until used.

9.4 Dose Levels: Selection of the dose levels in the mutagenesis assay was based upon toxicity as indicated by a decline in colony forming efficiency of the cells in the initial toxicity assay(s). Ideally, the high dose for the mutagenesis assay is selected to give a cell survival of 10 to 30%. However, since the test substance could not be delivered at a high enough concentration to be cytotoxic, the study was conducted with the maximum concentrations obtainable (100%). Precipitation of the test substance in the culture medium was not observed up to the highest dose tested.

9.4.1 Negative Control: The negative control consisted of cultures treated with 100% air only.

9.4.2 Positive Controls: Positive controls, both direct-acting and indirect-acting, were included to demonstrate the adequacy of the experimental conditions to detect known mutagens. Ethylmethanesulfonate (EMS) at 150 and 300 μg/ml was used as a direct-acting mutagen for the nonactivated portion, and dimethylnitrosamine (DMN) at 50 and 100 μg/ml was used as a

promutagen that requires metabolic activation. Both positive control substances were dissolved in dimethylsulfoxide and administered in a dosing volume of 50 µl per culture.

9.5 Identification: Using a permanent marking pen, all culturing and processing containers used in the study were uniquely identified with the ILS chemical number, S9 condition, dose, and date.

9.6 Type and Frequency of Tests:

9.6.1 Cytotoxicity Test: Cells seeded 18-24 hours earlier at 1.0×10^6 cells in 25 cm^2 flasks and in log phase when treated were exposed to solvent alone and 5 concentrations of the test substance in duplicate for 5 hours in the presence and absence of S9. Based on information provided by the sponsor, the concentrations evaluated in the initial toxicity assay were 10, 25, 50, 75, and 100 % FE-13, and 100% nitrogen (to determine the effects of oxygen deprivation). The osmolality of all treated and control cultures used in the toxicity assay was determined at the end of the exposure period using an osmometer. Also, a check of medium pH was performed by inspection using the phenol red indicator present in the culture media. The next day, the cells were trypsinized and plated in triplicate at a density of 200 cells per 60 mm dish. The relative and absolute CE were determined 10 days later. The cell survival of the treated groups is expressed relative to the solvent control group (relative cloning efficiency).

9.6.2 Mutagenesis Assay: Six concentrations of the test substance with and without S9 mix (plus concurrent solvent and positive controls) were used in the mutagenicity assay. The concentrations selected were based on the findings from the initial toxicity assay. Briefly, the concentrations were selected as follows: the high dose was the maximum obtainable dose. Five lower doses were selected, one which was known to be relatively non-toxic. Cells seeded 18-24 hours earlier were exposed to solvent alone and six concentrations of the test substance in duplicate for 5 hours at 37 ± 1°C in the presence and absence of S9 (day 0). After treatment, the tedlar bags were opened in a hood to vent the gas. The treatment medium was removed, the cells washed 2 times with Ca and Mg free Hanks Balanced Salt Solution (HBSS) and complete medium without additives was added for an additional 18-24 hours incubation.

9.6.2.1 Estimation of Cytotoxicity: Cytotoxicity determination was demonstrated by a lack of colony development. On day 1, 18-24 hours after the termination of treatment, flasks were subcultured, counted, and an

aliquot of AS52 cells seeded in triplicate at a density of 200 cells/60 mm dish. After 7-10 days incubation at 37 ± 1°C, colonies were fixed and stained, air dried, and counted. Cytotoxicity was expressed as relative CE (the ratio of the absolute CE of the treated cells to that of the solvent controls).

9.6.2.2 Phenotypic Expression: After mutation at the *gpt* locus, the mutant phenotype requires a period of time before it is completely expressed (expression requiring the loss of pre-existing enzyme activity). At the normal population doubling times of 12 to 16 hours for AS52 cells, an expression period of 6-7 days is required. On day 1, duplicate treatment flasks were trypsinized, counted, and an aliquot of AS52 cells seeded at a density of 1×10^6 cells per flask. Cells were subcultured on days 4 and 6 and selected for 6-TG resistance on day 6.

9.6.2.3 Mutant Selection: On day 6, plates from each treatment group were trypsinized, counted, and five replicate dishes plated at a density of 2×10^5 cells/100 mm dish in F-12 medium with 10 μM 6-TG. For cloning efficiency at the time of selection, 200 cells/60 mm dish were also plated in triplicate in F-12 medium without 6-TG. After 7-8 days of incubation at 37 ± 1°C, colonies were fixed, stained, and later counted for cloning efficiency and mutant selection.

9.7 Statistical Analysis: A decision to classify a mutagenic response as negative, equivocal, or positive must involve a consideration of the appropriateness of the concurrent control data, a formal statistical analysis of the experimental data, and interpretation as to the biological relevance of the response by an experienced scientific investigator. An alpha level of 0.05 was used to indicate statistical significance in all analyses. Due to the possibility of fluctuation, samples with less than 1×10^5 viable cells after treatment (i.e., $\leq$10% survival) were not considered as valid data points. Exact statistical analysis is difficult because the distribution of the number of mutant colonies depends on the complex processes of cell growth and death after mutagen treatment. While other appropriate methods can be used, the commonly used method is to use a one-tailed trend test, based on the number of mutant cells per 10^6 clonable cells in duplicate cultures, to evaluate for a positive increase in mutant frequency with increasing dose followed by a comparison of each treatment group against the concurrent solvent control treatment group. This pairwise comparison was made using student's t test, based on the number of mutant cells per 10^6 clonable cells, in duplicate cultures.

10.0 Criteria for Determination of a Valid Test

10.1 Negative Control: The absolute CE of the solvent controls should not be less than 60% and the mutant frequency of the negative controls in each experiment should fall within the range of 10 to 35 mutants per 10^6 clonable cells. Absolute CE values lower than 60% could indicate suboptimal culturing conditions for the cells while a higher mutant frequency may preclude detection of weak mutagens.

10.2 Positive Controls: The positive controls must induce a statistically significant response over the concurrent solvent control.

10.3 Test substance: The highest test substance concentration should, if possible, result in a significant cytotoxic response (e.g., 10% to 30% survival).

11.0 Criteria for a Positive Response

The response to the test substance will be deemed positive if a dose dependent increase in mutant frequency is observed with one or more of the six doses tested exhibiting a mutant frequency which is at least twice that of the negative control and is increased above the negative control by at least 10 mutants per million clonable cells.

If either, but not both, of the above conditions are met, the assay results will be evaluated by the study director and will be classified as positive, equivocal, or negative depending on the nature and magnitude of the response.

If neither of the above conditions are met, the test substance is classified as negative for mutagenic activity in this *in vitro* test.

12.0 Records to be Maintained:

Data were recorded on loose work sheets adapted or prepared as necessary for the test results. All data, stained plates, an original copy of the final report, and all correspondence will be archived at ILS until acceptance of the final report by the sponsor or three months after submission of the final report to the sponsor. This material will be made available to the sponsor upon request, and will not be discarded without written authorization from the sponsor.

13.0 Quality Assurance:

The protocol was reviewed by the ILS QAU before final approval. A quality assurance inspection of critical phases was conducted to assure the quality and integrity of the study results. An audit of the final report was conducted to determine the consistency between the reported information and the raw data.

14.0 Test Substance Disposition:

Any unused test substance and a log accounting for all test substance use will be returned to the sponsor upon completion of the contract.

15.0 Results:

15.1 Chemicals and Reagents: The chemicals and reagents used in this study (purity not provided) were obtained from the following commercial sources:

Chemical	Source	Lot No.
air	Air Products	N/A
calcium chloride	Sigma	84F-0723
dimethylsulfoxide	Fisher	933274
dimethylnitrosamine	Sigma	82H0365
ethylmethanesulfonate	Sigma	74H1107
fetal bovine serum	Irvine	300341227, 300240111
giemsa	J. T. Baker	J36550,
	Gurr	9368514
	Fisher	936851A
glucose-6-phosphate	Sigma	122H3786
glutamine	Irvine	931740438, 931750442
Ham's F-12	Irvine	905850826
Hank's balanced salt solution	Irvine	922850927
magnesium chloride	Sigma	44H0170
methanol	Fisher	952201, 963109
NADP	Sigma	114H7807
penn./strep./fung.	Gibco	19P4458, 20P6556
rat liver S9	Molecular Toxicology	0612
sodium phosphate	Aldrich	1326LL
thioguanine	Sigma	74F-0018

15.2 Toxicity Tests: Based on information provided by the sponsor, the concentrations evaluated in the initial toxicity assay were 10, 25, 50, 75, 100 % FE-13, and 100% nitrogen gas. Osmolality and pH data are presented in Table 1. Individual culture data, absolute CE, and relative CE are presented in Table 2.

15.2.1 Toxicity in Nonactivation Cultures: No significant change in media pH or osmolality was observed in the treated cultures. No decrease in the RCE was detected in treated cultures. Likewise, no toxicity was observed in the nitrogen control. Based on these results, the maximum dose of the test substance chosen to be tested in the absence of metabolic activation was selected to be 100% FE-13.

15.2.2 Toxicity in Activation Cultures: No significant change in media pH or osmolality was observed in the treated cultures. A dose dependent decrease in the RCE was detected in treated cultures. A mean RCE of 45% was observed at the highest dose, 100% FE-13. Based on these results, the maximum dose of the test substance to be tested in the presence of metabolic activation was selected to be 100 %.

15.3 Mutagenic Activity in the Absence of Metabolic Activation: AS52 cells were exposed in the absence of metabolic activation to FE-13 at 50, 60, 70, 80, 90, and 100 % FE-13 and 100% nitrogen gas. Due to unacceptably low cloning efficiency counts in the negative control cultures, the experiment was repeated over the same dose range. Individual and total plate counts as well as mutant frequency and induced mutant frequency data are presented in Table 4. The test substance did not induce a significant increase in mutant frequency (based on one million clonable cells), as demonstrated by a nonsignificant one-tailed trend test ($p = 0.638$) and the lack of a significant increase in mutant frequency at each dose group compared to the concurrent control ($p > 0.05$). The 100% nitrogen gas control was also not mutagenic ($p = 0.763$) compared to the mutant frequency of the negative controls. The positive control, EMS at 150 and 300 µg/ml, was mutagenic at both doses ($p < 0.05$) compared to the mutant frequency of the negative controls.

Concurrent cytotoxicity data and cloning efficiency data are presented in Tables 3 and 5, respectively. A depression in the RCE immediately following dosing was not observed among treated cultures, with a mean RCE of 95.6% observed at the top dose, 100% FE-13. The mean absolute cloning efficiency of the negative control cultures at the time of selection was 80.0%, well within the acceptable range.

15.4 Mutagenic Activity in the Presence of Metabolic Activation: AS52 cells were initially exposed in the presence of metabolic activation to FE-13 at 50, 60, 70, 80, 90, and 100 %, and nitrogen gas at 100%. Individual and total plate counts as well as mutant frequency and induced mutant frequency data are presented in Table 7. The test substance did not induce a significant increase in mutant frequency (based on one million clonable cells), as demonstrated by both a nonsignificant one-tailed trend test ($p = 0.067$) and the lack of a significant increase in mutant frequency at each dose group compared to the concurrent control ($p > 0.05$). The 100% nitrogen gas control was also not mutagenic ($p = 0.528$) compared to the mutant frequency of the negative controls. The positive control, DMN, was significantly mutagenic at 50 μg/ml ($p = 0.034$) but not 100 μg/ml ($p = 0.218$).

Concurrent cytotoxicity data and cloning efficiency data are presented in Tables 6 and 8, respectively. A significant depression in the RCE immediately following dosing was not observed among treated cultures, with the lowest mean depression of 69.3% observed at 80% FE-13. The mean absolute cloning efficiency of the negative control cultures at the time of selection was 65.4%, just above the acceptable range.

16.0 Conclusion

The test substance, FE-13 (ILS # 96-01), in either the presence or absence of metabolic activation did not induce a significant increase in the mutant frequency at the *gpt* locus in cultured AS52 cells.

17.0 References

(1) Stankowski, L.F. Jr., and K.R. Tindall (1987) Characterization of the AS52 cell line for use in mammalian cell mutagenesis studies. In: Banbury Report 28: Mammalian Cell Mutagenesis (M.M. Moore, D.M. DeMarini, F.J. de Serres, and K.R. Tindall, eds.), Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp. 71-79.

(2) Tindall, K.R., and L.F. Stankowski Jr. (1987) Deletion mutations are associated with the differential-induced mutant frequency response of the AS52 and CHO-K1-BH4 cell lines. In: Banbury Report 28: Mammalian Cell Mutagenesis (M.M. Moore, D.M. DeMarini, F.J. de Serres, and K.R. Tindall, eds.), Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp. 283-292.

(3) Aaron, C.S., G. Bolesfoldi, H.-R. Glatt, M. Moore, Y. Nishi, L. Stankowski, J. Theiss and E. Thompson (1994) International workshop on standardization of genotoxicity test procedures: Mammalian cell gene mutation assays working group report. Mutation Res. 312: 235-240.

TABLE 1: INITIAL TOXICITY OSMOLALITY AND pH IN AS52 CELLS TREATED WITH FE-13 (ILS # 96-01)

-S9				+S9			
DOSE (%)	CULTURE	OSMOL. (mOSMs)	pH	DOSE (%)	CULTURE	OSMOL. (mOSMs)	pH
100 air	A	280	N	100 air	A	262	N
	B	284	N		B	268	N
	MEAN	282			MEAN	265	
10.0	A	299	N	10.0	A	277	N
	B	293	N		B	276	N
	MEAN	296			MEAN	277	
25.0	A	289	N	25.0	A	281	N
	B	294	N		B	282	N
	MEAN	292			MEAN	282	
50.0	A	284	N	50.0	A	271	N
	B	286	N		B	273	N
	MEAN	285			MEAN	272	
75.0	A	297	N	75.0	A	272	N
	B	293	N		B	274	N
	MEAN	295			MEAN	273	
100.0	A	287	N	100.0	A	263	N
	B	292	N		B	285	N
	MEAN	290			MEAN	274	
100 N2	A	283	N	100 N2	A	257	N
	B	282	N		B	281	N
	MEAN	283			MEAN	269	

OSMOL = Osmolality in mOsms

N = Normal

N2 = nitrogen gas

TABLE 2: INITIAL TOXICITY TEST COUNTS FOR AS52 CELLS TREATED WITH FE-13 (ILS # 96-01)

DOSE (%)	FLASK	S9	PLATE COUNTS 1	2	3	MEAN	ACE	RCE
100 air	A	-	197	204	203	201.3	100.7	-
100 air	B	-	132	157	114	134.3	67.2	-
10.0	A	-	202	189	202	197.7	98.8	117.8
10.0	B	-	143	131	123	132.3	66.2	78.8
25.0	A	-	207	206	222	211.7	105.8	126.1
25.0	B	-	190	176	214	193.3	96.7	115.2
50.0	A	-	152	152	158	154.0	77.0	91.8
50.0	B	-	186	174	179	179.7	89.8	107.1
75.0	A	-	190	197	183	190.0	95.0	113.2
75.0	B	-	187	184	177	182.7	91.3	108.8
100.0	A	-	172	159	185	172.0	86.0	102.5
100.0	B	-	325	327	305	319.0	159.5	190.1
100 N2	A	-	188	221	176	195.0	97.5	116.2
100 N2	B	-	225	216	205	215.3	107.7	128.3
100 air	A	+	225	247	246	239.3	119.7	-
100 air	B	+	303	302	279	294.7	147.3	-
10.0	A	+	143	159	158	153.3	76.7	57.4
10.0	B	+	104	100	101	101.7	50.8	38.1
25.0	A	+	223	213	186	207.3	103.7	77.7
25.0	B	+	172	167	182	173.7	86.8	65.0
50.0	A	+	228	212	202	214.0	107.0	80.1
50.0	B	+	217	246	211	224.7	112.3	84.1
75.0	A	+	186	176	150	170.7	85.3	63.9
75.0	B	+	160	172	159	163.7	81.8	61.3
100.0	A	+	99	95	141	111.7	55.8	41.8
100.0	B	+	120	121	150	130.3	65.2	48.8
100 N2	A	+	223	235	249	235.7	117.8	88.3
100 N2	B	+	260	257	210	242.3	121.2	90.8

ACE = absolute cloning efficiency = mean plate count/200 cells*100

RCE = relative cloning efficiency = ACE dose/mean ACE solvent control*100

N2 = nitrogen gas

TABLE 3: CONCURRENT CYTOTOXICITY COUNTS FOR AS52 CELLS TREATED WITH FE-13 (ILS # 96-01)

DOSE			PLATE COUNTS					
(%)	FLASK	S9	1	2	3	MEAN	ACE	RCE
100 air	A	-	140	121	113	124.7	62.3	-
100 air	B	-	182	200	170	184.0	92.0	-
	MEAN					154.3	77.2	-
100 N2	A	-	160	172	173	168.3	84.2	109.1
100 N2	B	-	176	157	152	161.7	80.8	104.8
	MEAN					165.0	82.5	106.9
50	A	-	158	146	163	155.7	77.8	100.9
50	B	-	213	250	265	242.7	121.3	157.2
	MEAN					199.2	99.6	129.0
60	A	-	117	113	115	115.0	57.5	74.5
60	B	-	167	167	164	166.0	83.0	107.6
	MEAN					140.5	70.3	91.0
70	A	-	316	326	283	308.3	154.2	199.8
70	B	-	112	134	105	117.0	58.5	75.8
	MEAN					212.7	106.3	137.8
80	A	-	258	283	317	286.0	143.0	185.3
80	B	-	180	180	238	199.3	99.7	129.2
	MEAN					242.7	121.3	157.2
90	A	-	80	76	68	74.7	37.3	48.4
90	B	-	417	205	369	330.3	165.2	214.0
	MEAN					202.5	101.3	131.2
100	A	-	202	157	182	180.3	90.2	116.8
100	B	-	130	109	105	114.7	57.3	74.3
	MEAN					147.5	73.8	95.6
EMS 150	A	-	189	182	170	180.3	90.2	116.8
EMS 150	B	-	100	97	107	101.3	50.7	65.7
	MEAN					140.8	70.4	91.3
EMS 300	A	-	168	139	161	156.0	78.0	101.1
EMS 300	B	-	110	129	101	113.3	56.7	73.4
	MEAN					134.7	67.3	87.3

ACE = absolute cloning efficiency = (mean plate count/200 cells)*100

RCE = relative cloning efficiency = (ACE dose/mean ACE solvents)*100

N2 = nitrogen gas

TABLE 4: MUTANT SELECTION COUNTS FOR AS52 CELLS TREATED WITH FE-13 (ILS # 96-01)

DOSE			PLATE COUNTS							
(%)	FLASK	S9	1	2	3	4	5	TOTAL	MF	IMF
100 air	A	-	1	5	1	4	4	15	22.4	-
100 air	B	-	1	1	0	2	1	5	5.4	-
	MEAN								13.9	-
100 N2	A	-	1	0	0	2	2	5	11.1	-2.8
100 N2	B	-	0	2	2	5	3	12	24.1	10.2
	MEAN								17.6	3.7
50	A	-	1	1	3	5	3	13	13.2	-0.7
50	B	-	1	1	1	1	1	5	6.1	-7.8
	MEAN								9.6	-4.3
60	A	-	1	1	3	2	1	8	8.4	-5.4
60	B	-	1	0	0	0	0	1	1.0	-12.8
	MEAN								4.7	-9.1
70	A	-	3	1	2	3	2	11	20.6	6.7
70	B	-	1	1	0	2	1	5	9.0	-4.9
	MEAN								14.8	0.9
80	A	-	2	2	0	0	2	6	13.7	-0.2
80	B	-	3	1	3	3	2	12	19.4	5.5
	MEAN								16.5	2.6
90	A	-	2	3	1	3	2	11	12.7	-1.1
90	B	-	2	2	0	1	0	5	5.4	-8.5
	MEAN								9.1	-4.8
100	A	-	1	1	6	3	4	15	15.9	2.0
100	B	-	1	4	1	1	3	10	10.8	-3.1
	MEAN								13.3	-0.5
EMS 150	A	-	15	15	9	21	14	74	105.0	91.1
EMS 150	B	-	12	15	14	10	15	66	84.1	70.2
	MEAN								94.5	80.6 *
EMS 300	A	-	22	36	32	40	24	154	231.6	217.7
EMS 300	B	-	24	23	30	26	14	117	153.9	140.1
	MEAN								192.8	178.9 *

MF = Mutant Frequency per million clonable cells

IMF = Induced Mutant Frequency = MF dose - mean MF Solvent Controls

* = significantly different at $p < 0.05$

TABLE 5: CLONING EFFICIENCY COUNTS FOR AS52 CELLS TREATED WITH FE-13 (ILS # 96-01)

DOSE			PLATE COUNTS					
(%)	FLASK	S9	1	2	3	MEAN	ACE	RCE
100 air	A	-	130	135	137	134.0	67.0	-
100 air	B	-	215	178	165	186.0	93.0	-
	MEAN					160.0	80.0	-
100 N2	A	-	82	102	86	90.0	45.0	56.3
100 N2	B	-	100	98	101	99.7	49.8	62.3
	MEAN					94.8	47.4	59.3
50	A	-	183	200	210	197.7	98.8	123.5
50	B	-	159	165	168	164.0	82.0	102.5
	MEAN					180.8	90.4	113.0
60	A	-	186	190	193	189.7	94.8	118.5
60	B	-	164	206	202	190.7	95.3	119.2
	MEAN					190.2	95.1	118.9
70	A	-	104	108	109	107.0	53.5	66.9
70	B	-	122	109	102	111.0	55.5	69.4
	MEAN					109.0	54.5	68.1
80	A	-	77	96	90	87.7	43.8	54.8
80	B	-	122	120	130	124.0	62.0	77.5
	MEAN					105.8	52.9	66.1
90	A	-	173	188	157	172.7	86.3	107.9
90	B	-	198	187	168	184.3	92.2	115.2
	MEAN					178.5	89.3	111.6
100	A	-	178	196	192	188.7	94.3	117.9
100	B	-	176	198	182	185.3	92.7	115.8
	MEAN					187.0	93.5	116.9
EMS 150	A	-	122	146	155	141.0	70.5	88.1
EMS 150	B	-	166	129	176	157.0	78.5	98.1
	MEAN					149.0	74.5	93.1
EMS 300	A	-	152	113	134	133.0	66.5	83.1
EMS 300	B	-	147	156	153	152.0	76.0	95.0
	MEAN					142.5	71.3	89.1

ACE = absolute cloning efficiency = (mean plate count/200 cells)*100

RCE = relative cloning efficiency = (ACE dose/mean ACE solvents)*100

N2 = nitrogen gas

TABLE 6: CONCURRENT CYTOTOXICITY COUNTS FOR AS52 CELLS TREATED WITH FE-13 (ILS # 96-01)

DOSE			PLATE COUNTS					
(%)	FLASK	S9	1	2	3	MEAN	ACE	RCE
100 air	A	+	162	174	165	167.0	83.5	-
100 air	B	+	205	183	184	190.7	95.3	-
	MEAN					178.8	89.4	-
100 N2	A	+	135	125	101	120.3	60.2	67.3
100 N2	B	+	121	135	149	135.0	67.5	75.5
	MEAN					127.7	63.8	71.4
50	A	+	143	139	139	140.3	70.2	78.5
50	B	+	117	115	115	115.7	57.8	64.7
	MEAN					128.0	64.0	71.6
60	A	+	174	171	172	172.3	86.2	96.4
60	B	+	194	195	205	198.0	99.0	110.7
	MEAN					185.2	92.6	103.5
70	A	+	138	145	155	146.0	73.0	81.6
70	B	+	240	233	262	245.0	122.5	137.0
	MEAN					195.5	97.8	109.3
80	A	+	114	110	105	109.7	54.8	61.3
80	B	+	139	140	135	138.0	69.0	77.2
	MEAN					123.8	61.9	69.2
90	A	+	134	118	119	123.7	61.8	69.2
90	B	+	131	117	199	149.0	74.5	83.3
	MEAN					136.3	68.2	76.2
100	A	+	157	151	147	151.7	75.8	84.8
100	B	+	196	200	184	193.3	96.7	108.1
	MEAN					172.5	86.3	96.5
DMN 50	A	+	197	215	219	210.3	105.2	117.6
DMN 50	B	+	164	152	127	147.7	73.8	82.6
	MEAN					179.0	89.5	100.1
DMN 100	A	+	129	122	125	125.3	62.7	70.1
DMN 100	B	+	155	162	145	154.0	77.0	86.1
	MEAN					139.7	69.8	78.1

ACE = absolute cloning efficiency = (mean plate count/200 cells)*100

RCE = relative cloning efficiency = (ACE dose/mean ACE solvents)*100

N2 = nitrogen gas

TABLE 7: MUTANT SELECTION COUNTS FOR AS52 CELLS TREATED WITH FL-13 (ILS # 96-01)

DOSE			PLATE COUNTS							
(%)	FLASK	S9	1	2	3	4	5	TOTAL	MF	IMF
100 air	A	+	1	5	6	5	6	23	33.1	-
100 air	B	+	5	3	3	2	0	13	21.2	-
	MEAN								27.1	-
100 N2	A	+	1	6	2	3	3	15	12.4	-14.7
100 N2	B	+	14	2	6	5	3	30	27.3	0.2
	MEAN								19.9	-7.3
50	A	+	2	2	4	2	1	11	11.4	-15.7
50	B	+	7	7	3	7	4	28	24.1	-3.1
	MEAN								17.7	-9.4
60	A	+	1	0	1	1	1	4	4.8	-22.4
60	B	+	3	1	0	2	0	6	7.8	-19.3
	MEAN								6.3	-20.8
70	A	+	0	4	5	4	3	16	23.7	-3.4
70	B	+	2	2	2	3	0	9	15.5	-11.7
	MEAN								19.6	-7.6
80	A	+	2	5	3	1	2	13	15.5	-11.7
80	B	+	2	0	2	2	0	6	13.6	-13.6
	MEAN								14.5	-12.6
90	A	+	0	0	1	0	2	3	5.3	-21.9
90	B	+	1	3	3	1	1	9	9.9	-17.2
	MEAN								7.6	-19.6
100	A	+	2	1	2	3	3	11	14.1	-13.1
100	B	+	1	1	1	3	1	7	8.7	-18.4
	MEAN								11.4	-15.8
DMN 50	A	+	9	8	16	13	5	51	61.8	34.7
DMN 50	B	+	7	11	14	12	12	56	70.3	43.1
	MEAN								66.1	38.9 *
DMN 100	A	+	4	7	8	5	5	29	35.9	8.7
DMN 100	B	+	7	7	13	6	6	39	44.0	16.8
	MEAN								39.9	12.8

MF = Mutant Frequency per million clonable cells

IMF = Induced Mutant Frequency = MF dose - mean MF Solvent Controls

* = significantly different at $p < 0.05$

TABLE 8: CLONING EFFICIENCY COUNTS FOR AS52 CELLS TREATED WITH FE-13 (ILS # 96-01)

DOSE			PLATE COUNTS					
(%)	FLASK	S9	1	2	3	MEAN	ACE	RCE
100 air	A	+	124	139	154	139.0	69.5	-
100 air	B	+	147	92	129	122.7	61.3	-
	MEAN					130.8	65.4	-
100 N2	A	+	261	253	212	242.0	121.0	185.0
100 N2	B	+	217	237	205	219.7	109.8	167.9
	MEAN					230.8	115.4	176.4
50	A	+	207	186	185	192.7	96.3	147.3
50	B	+	246	242	210	232.7	116.3	177.8
	MEAN					212.7	106.3	162.5
60	A	+	171	161	169	167.0	83.5	127.6
60	B	+	156	147	158	153.7	76.8	117.5
	MEAN					160.3	80.2	122.5
70	A	+	142	135	128	135.0	67.5	103.2
70	B	+	114	113	122	116.3	58.2	88.9
	MEAN					125.7	62.8	96.1
80	A	+	189	157	158	168.0	84.0	128.4
80	B	+	71	98	96	88.3	44.2	67.5
	MEAN					128.2	64.1	98.0
90	A	+	116	120	105	113.7	56.8	86.9
90	B	+	204	171	170	181.7	90.8	138.9
	MEAN					147.7	73.8	112.9
100	A	+	160	152	157	156.3	78.2	119.5
100	B	+	171	151	160	160.7	80.3	122.8
	MEAN					158.5	79.3	121.1
DMN 50	A	+	155	153	187	165.0	82.5	126.1
DMN 50	B	+	155	151	172	159.3	79.7	121.8
	MEAN					162.2	81.1	123.9
DMN 100	A	+	179	171	135	161.7	80.8	123.6
DMN 100	B	+	179	168	185	177.3	88.7	135.5
	MEAN					169.5	84.8	129.6

ACE = absolute cloning efficiency = (mean plate count/200 cells)*100

RCE = relative cloning efficiency = (ACE dose/mean ACE solvents)*100

N2 = nitrogen gas

APPENDIX

PROTOCOL, AMENDMENTS, & DEVIATIONS

INTEGRATED LABORATORY SYSTEMS

STUDY PROTOCOL

1.0 Study Title:

AS52/GPT Mammalian Mutagenesis Assay

2.0 Study Identification:

Project No.: A073-003
Contract No. DAAD05-91-C-0018

3.0 Purpose of the Study:

To evaluate the ability of the test substance and/or its metabolites to induce gene mutations in the guanine phosphoribosyl-transferase (*gpt*) locus of cultured AS52 Chinese Hamster Ovary cells.

4.0 Names and Addresses of Sponsor and Testing Facility:

4.1	Sponsor:	U.S. Army CHPPM Bldg E-2100 Aberdeen Proving Ground, MD 21005
4.2	Testing Facility:	Integrated Laboratory Systems
	Shipping Address:	800-12 Capitola Drive Durham, NC 27713
	Mailing Address:	P.O. Box 13501 Research Triangle Park, NC 27709
4.3	Project Officer:	LeRoy Metker
4.4	Study Director:	Paul Andrews, M.S.

5.0 Proposed Study Dates:

Experimental Start Date: February 26, 1996
Experimental Termination Date: May 3, 1996

6.0 Test Substance:

6.1 Identification: FE-13 (ILS # 96-01)

6.2 Properties of the Test Substance:

6.2.1 Compound Characterization: Determination of test substance stability and test substance characteristics is the responsibility of the sponsor. Information on the test substance including its method of synthesis, analysis, physicochemical characteristics, and bulk stability is retained on file by the sponsor.

6.2.2 Storage Conditions: The test substance will be stored at room temperature in the ILS Chemical Repository. Stability under these conditions has been demonstrated by the sponsor and documentation is on file with them. Normal safety precautions appropriate for potential clastogens will be necessary when handling the test substance.

7.0 Test System:

7.1 Test System Justification: AS52 cells contain a single, functional, stably integrated copy of the *Escherichia coli* xanthine-guanine phosphoribosyl transferase (XPRT) gene (*gpt*). Mutations at the *gpt* locus can be detected as 6-thioguanine resistant (6-TG^r) colonies under conditions identical to those for detecting mutations at the *hprt* locus in CHO-K1-BH4 cells (1). However, in contrast to the *hprt* assay, the AS52/*gpt* assay is able to detect agents which induce primarily small and large deletion mutations in addition to point mutations (1,2). This ability to detect clastogens in addition to point mutagens results in an assay equal in sensitivity to the mouse lymphoma TK6$^{+/-}$ assay while retaining the technical simplicity of the CHO-K1-BH4 *hprt* assay (3). The AS52/*gpt* assay has been used to study a wide range of mutagens and clastogens, including radiation, a wide variety of chemicals, and complex mixtures.

7.2 AS52 Cell Line: The AS52 cell line, supplied by Dr. K.R. Tindall of the US National Institute of Environmental Health Sciences, is a proline auxotroph with a modal chromosome number of 20, a population doubling time of approximately 14 hours and a cloning efficiency normally greater than 80%. Cells are cultured in Ham's F-12 medium with 5% fetal bovine serum plus additives (xanthine, adenine, thymidine, mycophenolic acid, and aminopterin) at 37 ± 1°C in a humidified atmosphere of 5 ± 1% CO_2 in air.

8.0 Experimental Design:

8.1 Preparation of the Dosage Formulation: As specified by the Sponsor, test substance doses will be freshly mixed with air on the day of treatment.

8.2 Method of Administration and Justification: On the day of treatment, culture flasks are refed with a reduced volume of media (2.5 ml total) in order facilitate diffusion of the gaseous test substance to the cells. Flasks will then be sealed in plastic tedlar bags, the ambient air removed by vacuum, and the test substance doses introduced via a stainless steel valve stem.

8.3 S9 Activation System: Immediately prior to use, freshly thawed aliquots of Aroclor 1254-induced rat liver homogenates (S9 fraction) (Molecular Toxicology, Rockville, MD) will be mixed with a sterile cofactor pool. The S9 reaction mixture will be stored on ice until used.

8.4 Dose Levels: Selection of the dose levels in the mutagenesis assay will be based upon toxicity as indicated by a decline in colony forming efficiency of the cells in the initial toxicity assay(s). The high dose for the mutagenesis assay is selected to give a cell survival of 10 to 30%. In the event the test substance cannot be delivered at a high enough concentration to be cytotoxic, the study will be conducted with the maximum concentrations obtainable. Precipitation of the test substance in the culture medium will be permitted at the high dose only. Once the dose levels are determined, they will be added to the protocol in the form of an amendment.

8.4.1 Negative Control: The negative control will consist of cultures treated with 100% air.

8.4.2 Positive Controls: Positive controls, both direct-acting and indirect-acting, are included to demonstrate the adequacy of the experimental conditions to detect known mutagens. Ethyl methanesulfonate (EMS) at 150 and 300 μg/ml is used as a direct-acting mutagen for the nonactivated portion, and dimethylnitrosamine (DMN) at 150 and 300 μg/ml is used as a promutagen that requires metabolic activation. Both positive control substances will be dissolved in DMSO and administered in a dosing volume of 50 μl per culture.

8.5 Identification: Using a permanent marking pen, all culturing and processing containers used in the study will be uniquely identified with the ILS chemical number, S9 condition, dose, and date.

8.6 Type and Frequency of Tests:

8.6.1 Osmolality and pH Measurements: Mutagenicity can arise through nonspecific processes related to increased osmolality or to changes in pH (4). The osmolality of all treated and control cultures used in the toxicity assay will be determined at the end of the exposure period using an osmometer (Precision Systems). Also, a check of medium pH will be performed by inspection using the phenol red indicator present in the culture media unless precluded by an alteration in media color due to the test substance. If a substantial pH change occurs which can be detected visually, an aliquot will be removed and the pH quantitated using a pH meter. If a significant increase in osmolality (>400 mOsms) or a significant change in pH (<6.0 or >8.0) is detected at doses selected for mutagenicity testing, such measurements will also be taken in the mutagenicity tests and/or any repeat toxicity tests.

8.6.2 Cytotoxicity Test: Cells seeded 18-24 hours earlier at 1.0×10^6 cells in 25 cm^2 flasks and in log phase when treated will be refed with F-12 medium without serum or additives (2.5 ml total) and exposed to solvent alone and six concentrations of the test substance in duplicate for five hours in the presence and absence of S9. The nominal concentrations evaluated in the initial toxicity assay will be 100, 75, 50, 25, 10, and 5.0 % FE-13, 100% air, and 100% nitrogen (to determine the effects of oxygen deprivation). After treatment, the tedlar bags will opened in a hood to vent the gas. Treatment medium will be removed, the cells washed 2 times with Ca and Mg free Hanks Balanced Salt Solution (HBSS) and complete medium without additives added for an additional 18-24 hours incubation. The next day, the cells are trypsinized and plated in triplicate at a density of 200 cells per 60 mm dish. The relative and absolute CE will be determined 7-10 days later. The cell survival of the treated groups is expressed relative to the negative control group (relative cloning efficiency).

8.6.3 Mutagenesis Assay: Six concentrations of the test substance with and without S9 mix (plus concurrent solvent and positive controls) will be used in the mutagenicity assay. The concentrations selected will be based on the findings from the initial toxicity assay. Briefly, the concentrations will be selected as follows: the high dose is selected to give a cell survival of 10 to 30%. Five lower doses are selected, one which is known to be non-toxic. If the test substance cannot be mixed at a high enough concentration in the solvent to be cytotoxic or if excessive precipitation

occurs in the culture medium, the study will be conducted at the maximum concentration obtainable. Cells seeded 18-24 hours earlier will be refed with F-12 medium without serum or additives and exposed to air alone and to six concentrations of the test substance in duplicate for five hours at 37 ± 1°C in the presence and absence of S9 (day 0). Dilutions of the positive controls are prepared and 50 µl aliquots added to each flask containing 5 ml culture medium. After treatment, the tedlar bags will opened in a hood to vent the gas. Treatment medium will be removed, the cells washed 2 times with HBSS, and complete medium without additives added for an additional 18-24 hours incubation.

8.6.3.1 Estimation of Cytotoxicity: Cytotoxicity determination is demonstrated by a lack of colony development. On day 1, 18-24 hours after the termination of treatment, flasks are subcultured, counted, and an aliquot of AS52 cells seeded in triplicate at a density of 200 cells/60 mm dish. After 7-10 days incubation at 37 ± 1°C, colonies are fixed and stained in 50% methanol/4% Giemsa for approximately 5 minutes, air dried, and counted. Cytotoxicity is expressed as relative CE (the ratio of the absolute CE of the treated cells to that of the solvent controls).

8.6.3.2 Phenotypic Expression: After mutation at the *gpt* locus, the mutant phenotype requires a period of time before it is completely expressed (expression requiring the loss of pre-existing enzyme activity). At the normal population doubling times of 12 to 16 hrs for AS52 cells, an expression period of 6-7 days is required. On day 1, duplicate treatment flasks are trypsinized, counted, and an aliquot of AS52 cells seeded at a density of 1×10^6 cells per flask in F-12 medium plus fetal bovine serum without additives. Cells are subcultured on days 4 and 6 and selected for 6-TG resistance on day 6.

8.6.3.3 Mutant Selection: On day 6, plates from each treatment group are trypsinized, counted, and five replicate dishes are plated at a density of 2×10^5 cells/100 mm dish in F-12 medium with 10 µM 6-TG. For cloning efficiency at the time of selection, 200 cells/60 mm dish are also plated in triplicate in F-12 medium without 6-TG. After 7-10 days of incubation at 37 ± 1°C, colonies are fixed and stained as

previously described and counted for cloning efficiency and mutant selection. The spontaneous mutant frequency should be 10-35 x 10^{-6}. Negative controls should yield an absolute cloning efficiency of 60% or greater.

8.7 Data Presentation: Results from the mutagenesis assay will include the following experimental data:

8.7.1. Absolute and relative cloning efficiencies (CE) in the concurrent cytotoxicity assay, where:

Absolute CE = # colonies formed/# cells plated
Relative CE = CE (treatment)/CE (solvent)

8.7.2 Actual number of mutant colonies observed for each treatment condition.

8.7.3 Mutant frequency (MF) values, where:

MF = # mutant colonies / # clonable cells, where:

clonable cells = # cells plated x absolute CE at selection.

(MF is expressed as # mutants per 1 x 10^6 clonable cells)

8.7.4 Absolute CE at mutant selection for each treatment condition.

8.8 Statistical Analysis: A decision to classify a mutagenic response as negative, equivocal, or positive must involve a consideration of the appropriateness of the concurrent control data, a formal statistical analysis of the experimental data, and interpretation as to the biological relevance of the response by an experienced scientific investigator. An alpha level of 0.05 will be used to indicate statistical significance in all analyses. Due to the possibility of fluctuation, samples with less than 1 x 10^5 viable cells after treatment (i.e., $\leq$10% survival) are not considered as valid data points. Exact statistical analysis is difficult because the distribution of the number of mutant colonies depends on the complex processes of cell growth and death after mutagen treatment. While other appropriate methods can be used, one commonly used method is to use a one-tailed trend test to evaluate for a positive increase in mutant frequency with increasing dose followed by a comparison of each treatment group against the concurrent negative control treatment group.

9.0 Criteria for Determination of a Valid Test

9.1 Negative Control: The absolute CE of the negative controls should not be less than 60% and the mean mutant frequency of the negative controls in each experiment should fall within the range of 10 to 35 mutants per 10^6 clonable cells. Absolute CE values lower than 60% would indicate suboptimal culturing conditions for the cells while a higher mutant frequency may preclude detection of weak mutagens.

9.2 Positive Controls: The positive controls must induce a statistically significant response over the concurrent solvent control.

9.3 Test Substance: The highest test substance concentration should, if possible, result in a significant cytotoxic response (e.g., 10% to 30% survival). This is particularly important if the response is negative.

10.0 Criteria for a Positive Response

The response to the test substance will be deemed positive if a dose dependent increase in mutant frequency is observed with one or more of the six doses tested exhibiting a mutant frequency which is at least twice that of the negative control and is increased above the negative control by at least 10 mutants per million clonable cells.

If either, but not both, of the above conditions are met, the assay results will be evaluated by the Study Director and be classified as positive, equivocal, or negative depending on the nature and magnitude of the response.

If neither of the above conditions are met, the test substance is classified as negative for clastogenic activity in this *in vitro* test.

11.0 Records to be Maintained:

Data will be recorded on loose work sheets adapted or prepared as necessary for the test results. The experimental data, stained dishes, and data analysis diskettes are archived at ILS at the completion of the study until acceptance of the final report by the sponsor or three months after submission of the final report to the sponsor. After this time all items will be transferred to the sponsor for archiving.

12.0 Report:

The final report will contain a summary of the experimental design, materials, test procedures, and results. Data are presented in tabular form for the relative toxic effects of treatment, the total number of mutant colonies, and the mutant frequency per dose group.

13.0 Good Laboratory Practices Compliance:

The study will be conducted in accordance with Good Laboratory Practice regulations as promulgated by the U.S. Environmental Protection Agency (40 CFR Part 792). The protocol will be reviewed by the ILS Quality Assurance Unit before final approval. A quality assurance inspection of critical phases will be conducted to assure the quality and integrity of the study results. An audit of the final report will be conducted to determine the consistency between the reported information and the raw data. An appropriate QA statement will be added to the final report.

14.0 Personnel Health and Safety:

Safety procedures will be adhered to as stated in the ILS Health and Safety Manual and standard operating procedures. At the termination of the study, all waste materials will be disposed of in those areas specifically designed for the disposal of hazardous substances.

15.0 Test Substance Disposition:

All unused test substance along with a log accounting for its use will be returned to the sponsor upon completion of the contract.

16.0 References:

(1) Stankowski, L.F. Jr., and K.R. Tindall (1987) Characterization of the AS52 cell line for use in mammalian cell mutagenesis studies. In: Banbury Report 28: Mammalian Cell Mutagenesis (M.M. Moore, D.M. DeMarini, F.J. de Serres, and K.R. Tindall, eds.), Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp. 71-79.

(2) Tindall, K.R., and L.F. Stankowski Jr. (1987) Deletion mutations are associated with the differential-induced mutant frequency response of the AS52 and CHO-K1-BH4 cell

lines. In: Banbury Report 28: Mammalian Cell Mutagenesis (M.M. Moore, D.M. DeMarini, F.J. de Serres, and K.R. Tindall, eds.), Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp. 283-292.

(3) Aaron, C.S., G. Bolesfoldi, H.-R. Glatt, M. Moore, Y. Nishi, L. Stankowski, J. Theiss and E. Thompson (1994) International workshop on standardization of genotoxicity test procedures: Mammalian cell gene mutation assays working group report. Mutation Res. 312: 235-240.

(4) Scott, D., S.M. Galloway, R.R. Marshall, M. Ishidate, D. Brusick, J. Ashby, and B. Myhr (1991) Genotoxicity under extreme culture conditions. A report from ICPEMC Task Group 9. Mutation Res. 257: 147-204.

17.0 Approvals

Sponsor (Project Officer): LeRoy W. Hulstad Date: 2/22/96

Study Director: Paul Goodwin Date: 2/20/96

INTEGRATED LABORATORY SYSTEMS

PROTOCOL AMENDMENT

SPONSOR CODE: A1 PROTOCOL AMENDMENT NO.: 2 | *

ILS PROJECT NO.: A073-003 CHEMICAL REPOSITORY #: 96-01

STUDY TITLE: AS52/GPT Mammalian Mutagenesis Assay

Change Made:

Based on the data collected from the initial toxicity test, the following six doses were selected for the mutation assay in both the absence and presence of metabolic activation:

100, 90, 80, 70, 60, and 50 % FE-13, 100% air, and 100% nitrogen.

Section Changed:

8.4 Dose Levels

Reason:

GLP Requirement.

Submitted by:

Paul Goodman
Study Director

3/5/96
Date

* (16) PMA 5-7-96

INTEGRATED LABORATORY SYSTEMS

PROTOCOL AMENDMENT

SPONSOR CODE: A1 PROTOCOL AMENDMENT NO.: 2 *

ILS PROJECT NO.: A073-003 CHEMICAL REPOSITORY #: 96-01

STUDY TITLE: AS52/GPT Mammalian Mutagenesis Assay

Change Made:

Based on the data collected from the first mutation assay in the absence of metabolic activation, the test will repeated without S9 over the same dose range:

100, 90, 80, 70, 60, and 50 % FE-13, 100% air, and 100% nitrogen.

Section Changed:

8.4 Dose Levels

Reason:

The cloning efficiency of the negative control cultures was below the acceptable range.

Submitted by:

P. Gupta

Study Director

4/3/96

Date

* (16) AmA 5-7-96

INTEGRATED LABORATORY SYSTEMS

PROTOCOL DEVIATION

SPONSOR CODE: A1 PROTOCOL DEVIATION #: 1

ILS PROJECT NO.: A073-003 CHEMICAL REPOSITORY #: 96-01

STUDY TITLE: AS52/GPT Mammalian Mutagenesis Assay

Deviation:

The 5.0 % FE-13 dose was dropped from the toxicity test both with and without S9.

Cause of the Deviation:

The dose was dropped due to a limited number of tedlar bags and the expected low toxicity of the test substance. The Study Director did not write a protocol amendment before the action was taken.

Corrective Action Taken:

None

Impact on Study:

None

Submitted by:

Paul Caldwell — Study Director

5/7/96 — Date

INTEGRATED LABORATORY SYSTEMS

PROTOCOL DEVIATION

SPONSOR CODE: A1 PROTOCOL DEVIATION #: 2

ILS PROJECT NO.: A073-003 CHEMICAL REPOSITORY #: 96-01

STUDY TITLE: AS52/GPT Mammalian Mutagenesis Assay

Deviation:

The doses of the positive control, DMN, used in the mutation assay with S9 activation were 50 and 100 ug/ml.

Cause of the Deviation:

The DMN doses were mistakenly listed as 150 and 300 ug/ml in the study protocol.

Corrective Action Taken:

None

Impact on Study:

None

Submitted by:

P. Carolus 5/10/96

Study Director Date

INTEGRATED LABORATORY SYSTEMS

PROTOCOL DEVIATION

SPONSOR CODE: A1 PROTOCOL DEVIATION #: 3

ILS PROJECT NO.: A073-003 CHEMICAL REPOSITORY #: 96-01

STUDY TITLE: AS52/GPT Mammalian Mutagenesis Assay

Deviation:

The FE-13 tank was stored in the treatment lab rather than the chemical repository for the duration of the study.

Cause of the Deviation:

Due to the extensive rotometer connections necessary for dosing a gas, the tank was left in the treatment lab for logistical reasons, chained to a tank carrier.

Corrective Action Taken:

None

Impact on Study:

None

Submitted by:

P. Carolus

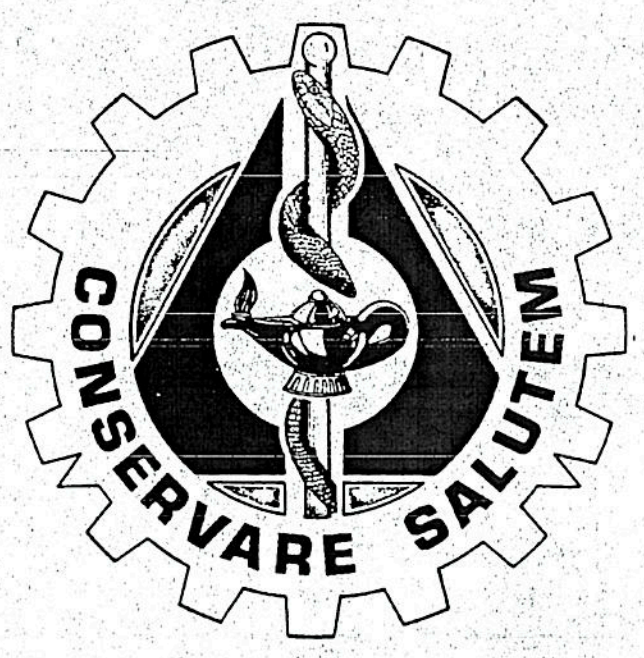
Study Director

5/10/96

Date

USACHPPM

U.S. Army Center for Health Promotion and Preventive Medicine



Mutagenicity Testing of FE-13

Readiness Thru Health

Executive Summary

Since the fire extinguishant, Halon 1301 poses an atmospheric ozone depletion potential and environmental regulations no longer allow its production, a suitable replacement must be found. One product currently under examination as a replacement for Halon 1301 is FE-13. (Freon 23; trifluromethane; CHF_3) is a halogenated hydrocarbon considered to be chemically inert although it can release fluoride when exposed to flame or red-hot metal. The median lethal concentration (LC_{50}) of FE-13, based on a 4-hour exposure, is >650,000 ppm. A Toxicity Profile developed for The Army Program Executive Office, Armored Systems Modernization by the Toxicology Division, AEHA in 1994 indicated that no effects were observed for FE-13 in 90-day exposure regimes at 10,000 ppm (1%). The effective extinguishant concentration of FE-13 is 12%. The no observable adverse effects level (NOAEL), based on cardiac sensitization, for FE-13 is greater than 30%. The Toxicity Profile indicated that further testing was necessary to determine developmental, reproductive and mutagenicity potential.

Genotoxic testing is an important component of a toxicological profile. Compounds which induce alterations in nucleic acids and associated components are considered to be genotoxic. Mutagenic testing is a specific type of genotoxic testing. Mutagens can induce types of stable changes in the nucleotide sequence of genes, the chromosome structure, or the chromosome number. These types of genetic events are responsible for a large proportion of human genetic diseases and congenital defects.

The compound FE-13 was tested for its mutagenic potential using four separate test systems. Each test system examined a specific mutagenic component. These test procedures included both *in vivo* and *in vitro* assays.

The AS52/GPT mammalian mutagenesis assay examines a compound's ability to induce gene mutations in the genes which code for the enzyme guanine phosphoribosyl-transferase (*gpt*) of cultured AS52 Chinese hamster ovary cells[1]. The addition of the metabolic activator, S9, allows the identification of promutagens. This test procedure is capable of identifying agents which cause small and large deletion mutations as well as point mutations. Also, this assay can demonstrate the cytotoxicity of the compound by comparing the cloning efficiency of treated cultures with that of nontreated cultures. Cultures were exposed to air concentrations of FE-13 for five hours at 37°C. Concentrations of FE-13, with and without the S9 activator, were 10, 25, 50, 75, and 100%. Some cultures were exposed to 100% nitrogen in order to determine the effects of oxygen deprivation. FE-13 did not induce a significant level of mutagenicity in the presence or absence of metabolic activation.

The *Salmonella typhimurium* / microsome reverse mutation assay (Ames test), developed by Bruce Ames, is an elegant assay for the determination of mutagenicity[2]. This assay employs bacterial strains that are unable to manufacture histadine and is capable of detecting both base pair substitutions and frameshift mutations. The metabolic activator, S9, is used in this test to identify promutagens. The concentrations of FE-13 used in this test procedure were 10, 50, and 100% per plate. FE-13 did not induce a significant level of mutagenicity in the presence or absence of metabolic activation.

In vitro Chromosome aberrations can also be examined using Chinese hamster ovary (CHO) cells[1]. This assay is sensitive to clastogenic activity of a variety of chemicals. The

detection of a significantly elevated level of chromosome damage is considered an indicator of genetic damage. The S9 fraction of rat liver homogenate is also used in this test system to identify promutagens. Toxicity of FE-13 was examined in cultures, with and without S9, using concentrations of 5, 10, 25, 50, 75, and 100% and a four hour exposure period. One group was exposed to 100% nitrogen in order to determine the effects of oxygen deprivation. This procedure examined average generation time, mitotic index, polyploid index, and cell density. Clastogenic activity was evaluated using concentrations of 50, 60, 70, 80, 90, and 100% in the presence or absence of the S9 activator. As with the toxicity portion of this study, one group was exposed to 100% nitrogen in order to determine the effects of oxygen deprivation. A continuous exposure protocol in the absence of S9 was not practical due to the potential adverse effects of oxygen deprivation. FE-13 was found to induce a significant level of clastogenic damage in concentrations of 80% and above in the absence of metabolic activation. Control (air only) cultures containing S9 displayed a 2% (not statistically significant) increase in cellular damage while nonactivated cultures displayed no damage. This difference in baseline activity may have accounted for the nonsignificant increase in cellular damage with the S9 activator although the level of damage from exposure was identical with and without the S9 activator. Cells exposed to 100% nitrogen also displayed the same level of damage. Damage, therefore, is probably due to a decreased oxygen level rather than the activity of FE-13.

The mouse bone micronucleus assay is an *in vivo* test system which can determine the ability of a compound to induce micronuclei formation in immature erythrocytes of male and female mice[3]. Micronuclei are formed when chromosomes lag or fragment during cell division. The B6C3F1 strain of mouse was used in this study as this strain appears to be exquisitely sensitive to micronucleus induction. This assay is the most reliable method for evaluating the potential of a compound to induce clastogenic or aneugenic damage. FE-13 was assayed using concentrations of 13%, 26%, 50%. Control animals were exposed to 100% air as well as an oxygen poor environment of 50% air and 50% nitrogen. FE-13 did not induce a significant level of mutagenicity.

The results of the above tests indicate that FE-13 does not induce a mutagenic effect at dosage levels tested and, from a mutagenicity standpoint, it appears to be a suitable replacement for Halon 1301. Further genotoxicity testing of this material is not indicated at this time.

References

1. Preston, R.J., W. Au, M.A. Bender, J.G. Brewen, A.V. Carrano, J.A. Heddle, A.F. McFee, S. Wolf, and J.S. Wassom. (1981). Mammalian *in vivo* and *in vitro* cytogenic assays: A report of the U.S.E.P.A. Gen-Tox Program. Mutation Res. 87:143-188.

2. Ames, B.N., J. McCann, and E. Yamasaki. (1975). Methods for detecting carcinogens and mutagens with the Salmonella mammalian microsome mutagenicity chromosome. Mutation Res. 31:347-364.

3. Heddle, J.A., M. Hite, B. Kirkhart, K. Mavrournin, J.T. MacGregor, G.W. Newell, and M.F. Salamone. (1983). The induction of micronuclei as a measure of genotoxicity. A report of the U.S.E.P.A. Gene-Tox Program. Mutation Res. 123:61-118.

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